

Effects of Wounding on Taxol and Baccatin III Levels in *Taxus media* cv *Hicksii*¹

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ABSTRACT. Two experiments were carried out to test the hypothesis that herbivory induces higher levels of taxanes in the bark of two year old *Taxus media* cv *Hicksii* cuttings. For the first experiment bark was cut 1.0 mm deep every 5.0 mm in two groups of cuttings; with 0.1% 2,4-D applied over the wounded bark in one group. Both groups and a control were harvested one week later. In the second experiment bark was wounded (as above) and treated cuttings with their respective control groups analyzed one and three weeks after treatment. Bark extracts were analyzed by high performance liquid chromatography.

Cutting significantly decreased taxol levels in the second, but not the first, experiment. Cutting tended to increase baccatin III in both experiments, though not significantly. Application of 2,4-D decreased baccatin III, but did not significantly alter the taxol concentrations. These results suggest that taxol and baccatin III are not induced after wounding *Taxus* bark, or that the time course of induction is shorter than three weeks.

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INTRODUCTION

Many secondary compounds produced by plants are inducible, that is their concentration in all or part of the plant increases in response to an external stimulus such as herbivory (Lewison et al. 1991a, Lewison et al. 1991b). However, it is not known whether the medically important taxanes are inducible in *Taxus* spp. (yews). These taxanes include taxol, which is approximately 30% effective in ovarian cancer patients (Stone 1993) and baccatin III, which is used in the hemi-synthesis of taxol (Bombardelli et al. 1992).

We hypothesized that taxanes are inducible defenses against herbivory or pathogens in yews, in part because other diterpenes have been shown to deter herbivory (Robinson 1991) and to be inducible in conifers (Rosenthal and Berenbaum 1991). *Taxus* spp. are subject to little insect herbivory, perhaps due to deterrence by taxanes (Perusse et al. 1977).

We further hypothesized that such induction can result from mechanical injury of the bark. This hypothesis is based on studies that showed terpene concentration increased after wounding in diverse taxa (Kahl 1978). Furthermore, the activity of monoterpene cyclase (an enzyme responsible for the formation of terpenes) increased seven days after mechanical wounding in three species of *Abies* and one species of *Picea* (Lewison et al. 1991a). This is important because Lewison et al. (1991b) found a direct correlation between the amount of monoterpene cyclase activity and monoterpene content.

We also hypothesized that taxane production can be increased through mechanical wounding of the bark coupled with the application of 2,4-dichlorophenoxyacetic acid (2,4-D). Fahn and Zamski (1970) demonstrated that 2,4-D in lanolin was effective at inducing resin flow and

increasing the number of vertical resin ducts and tracheids in *P. halepensis*. Although *Taxus* spp. lack normal and traumatic resin canals and vertical tracheids where resins are stored (Brown and Panshin 1940), it still may be possible that 2,4-D may induce proliferation of taxane-producing cells.

MATERIALS AND METHODS

Plant Material

Taxus media cv *Hicksii* (Nuttall) was used for both experiments because it was shown to have similar or higher amounts of taxol than *T. brevifolia* (Vidensek et al. 1990). Cuttings which had been rooted for two years were donated by Kern Nursery (Liberty Township, OH; Butler Co.). Cuttings for both experiments were about 35 cm tall and moved to a greenhouse 12 weeks prior to treatment. The cuttings for the 1993 experiment were moved to a greenhouse on March 22, treated June 8, and harvested June 15. The mean low and high temperatures were 15° and 32° C, respectively, for the 1993 experiment. The rooted cuttings for the 1994 experiment were moved to the greenhouse January 13, treated April 7, and harvested April 14 and 28. The mean low and high temperatures for 1994 were 13° and 24° C. Plants were fertilized with Peter's Solution® (Milpitas, CA) (N20 P10 K20) as needed (up to two times daily) with a natural photoperiod. Voucher specimens were deposited in the Willard Sherman Turrell Herbarium (MU) of Miami University.

Treatments

Experiment One (1993)

One hundred seventeen rooted cuttings were assigned to three treatment groups using a random number table. Unfortunately, due to centrifuge tubes cracking, only 13 control, 16 cut, and 34 cut and 2,4-D treated samples were analyzed. For treatment one, a 5.0 mm horizontal cut through the bark was made every 5.0 mm from the soil level to where the bark was approximately half

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woody. Each cut was approximately 25% around the stem. Wounding was done with a new razor blade for each plant, which was sanitized with 99.9+% methanol. In treatment two, the bark was cut into as above and a 0.1% concentration of 2,4-D in lanolin applied to the trunk with a brush. Treatment three, the control group, was not cut with a razor blade, or treated with 2,4-D. The trees were grown for one week after treatment as this time interval was shown to be sufficient for the amount of monoterpene cyclase activity to increase 5-15 fold (Lewison et al. 1991b), then cut off at soil level and immediately stripped of their bark.

Experiment Two (1994)

In 1994, 120 rooted cuttings were randomly assigned to four groups. Trees in two groups were cut with a razor blade as in the 1993 experiment; one group was allowed to grow for one week after treatment; the second, three weeks. The other two groups were controls that were harvested synchronously with their respective treatment group. No 2,4-D was used in any of these treatments. Stems were cut and stripped of their bark as in 1993.

Extraction Procedure

Bark samples were dried at 23° C then ground in liquid nitrogen with a mortar and pestle. Taxanes were extracted in methanol by sonication for 30 minutes followed by centrifugation for 10 minutes. Each bark sample was extracted three times; the three extracts were pooled and concentrated by evaporating off methanol with nitrogen while heating to 40° C. The residue in each of the centrifuge tubes was dissolved in 1.5 ml methanol:water (30:70) and purified through Prepsep™ C-18 columns under the vacuum of a 60 cc syringe. This procedure followed Auriola et al. (1992).

Analysis

The High Performance Liquid Chromatography (HPLC) conditions followed Auriola et al. (1992). The HPLC system used to analyze the samples included a Scientific Systems Inc. model 300 LC pump and a Lo-Pulse® model LP-21 pulse dampener used to propel the solvent (mobile phase) at a steady rate of 0.2 ml/min. A Rheodyne® injector equipped with a 20 µL sample loop delivered the sample to the column. A Phenomenex® Curosil-G-6µ model 03A-3121-G0 guard column (30 x 4.6 mm) and model 00G-3121-R0 analytical column (250 x 3.2 mm) were employed to separate the taxanes. The first experiment used a Waters® 490 E programmable multiwavelength detector to measure amounts of taxanes, while the second experiment employed a LDC® SpectroMonitor III model 1204 A detector. Both detectors were set at 228 nm. Both experiments employed a Linear® 1200 strip chart recorder, and the second experiment also employed a Shimadzu® Chromatopac C-R6A electronic integrator to automatically measure peak area. Statistical analysis was accomplished using SAS (1990). More specific methods are provided by Egan (1994).

RESULTS

Experiment One (1993)

Taxol levels did not differ among the treatments (Table 1). A one way ANOVA demonstrated a significant effect of treatment on baccatin III concentrations (Fig. 1, Table 1). Based on Bonferroni multiple comparison procedure, the only significant pairwise difference was between the cut treatment and the treatment in which the bark was cut then treated with 2, 4-D. There was a 45% increase in baccatin III for the cut treatment in relation to the control group, and a 56% decrease in the cut, 2,4-D treatment compared to the control group.

TABLE 1

One way ANOVA of percent taxol and baccatin III on treatment (control, cut, and cut with 2,4-D) in 1993.

Source	DF	Sum of Squares	F	Prob
Taxol				
Treatment	2	0.0005	1.73	0.1924
Error	36	0.0052		
Total	38	0.0057		
Baccatin III				
Treatment	2	0.00015	5.28	0.0102
Error	33	0.00046		
Total	35	0.00061		

Experiment Two (1994)

Two way analysis of variance (ANOVA) showed that there was a statistically significant change in taxol levels due to cutting the bark at weeks one or three (Fig. 2, Table 2).

A two way ANOVA showed that neither cutting the bark nor week of harvest significantly affected the percent of baccatin III in the bark (Table 2). The interaction

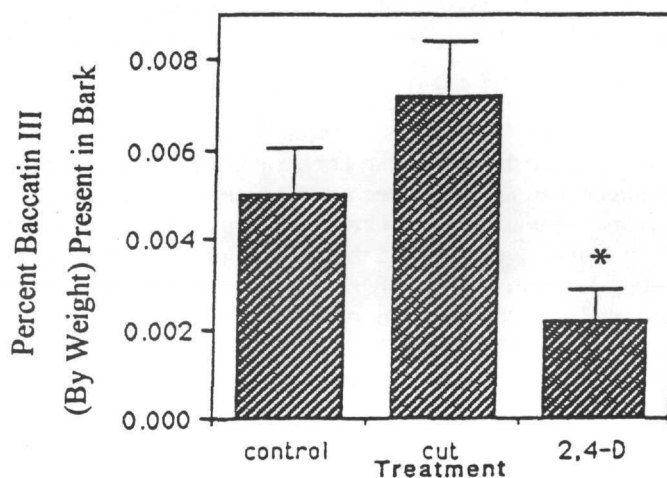


FIGURE 1. Percent baccatin III in the bark (mean + standard error) for the control and experimental groups in the 1993 experiment.

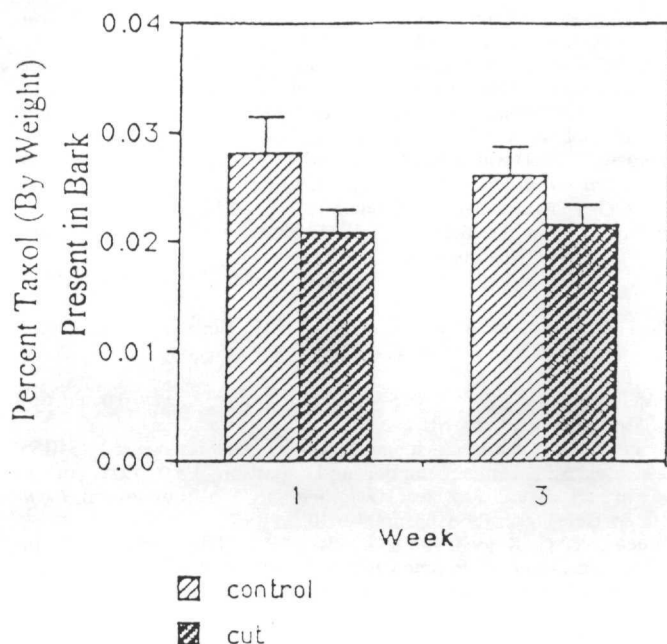


FIGURE 2. Percent taxol in the bark (mean + standard error) for the control and cut groups harvested one and three weeks after cutting for the 1994 experiment.

of cutting and time after cutting also was not statistically significant. For trees harvested one week after treatment the means of the control and cut treatments were not significantly different ($T = 0.63$, $P = 0.53$). For trees harvested three weeks after treatment the difference between the means approached statistical significance ($T = 1.94$, $P = 0.06$), with the cut trees averaging more baccatin III.

DISCUSSION

Effects of Wounding on Taxol

Treatments in the 1993 and 1994 experiments did not significantly increase taxol, therefore we cannot accept our hypothesis that wounding the bark increases taxol.

TABLE 2

Two way ANOVA for percent taxol and baccatin III on treatment (control vs. cut) and week of harvest for the 1994 experiment.

Source	DF	Type I SS	F	Prob
Taxol				
Treatment	1	0.00080	5.20	0.0250
Week	1	0.00001	0.07	0.7860
Treatment * Week	1	0.00004	0.25	0.6173
Error	89	0.01364		
Total	92	0.01449		
Baccatin III				
Treatment	1	0.000087	2.47	0.1193
Week	1	0.000003	0.07	0.7907
Treatment * Week	1	0.000015	0.42	0.5207
Error	89	0.003146		
Total	92	0.003250		

The different responses of *T. media* cv *Hicksii* to cutting in the two years may be due to seasonal patterns of taxol synthesis (Wheeler et al. 1992). The 1993 cuttings were moved to the greenhouse in March, and the treatments and harvesting were performed in June. The 1994 cuttings were moved in January, and the treatments and harvesting were done in April. Trees in the latter experiment may have been more actively synthesizing taxol and cutting may have disrupted taxol synthesis. The levels of taxol in the 1994 controls averaged slightly higher than the 1993 controls possibly due to the cooler greenhouse temperatures and less daylight. These conditions may favor taxol synthesis as *T. brevifolia* growing in cool, moist, and shaded areas have higher taxol levels (Wheeler et al. 1992).

Effects of Wounding on Baccatin III

Wounding had no significant effect on baccatin III for either the 1993 or the 1994 experiment. It is possible that too much time had passed and taxol levels returned to pre-wound levels. It is conceivable that baccatin III, but not taxol, increases in concentration after wounding because baccatin III may serve as a building block for taxol. Because baccatin III is used as a precursor molecule for hemi-synthesis of taxol *in vitro* (Bombardelli et al. 1992), it is possible that baccatin III serves as a precursor to taxol *in vivo*. Some induced defenses are initiated in as little as hours after wounding (Fritz and Simms 1992), so it is possible that taxol concentrations returned to non-induced concentrations in the bark before sampling at one and three weeks.

Effects of 2,4-D

In the 1993 experiment 2,4-D decreased the levels of taxol and baccatin III in the yew bark which was contrary to our hypothesis. Pollard and Walker (1990) suggest that 2,4-D in growth media may prevent some callose from undergoing differentiation. Gibson et al. (1993) observed that although the best nutrient media for yew callose growth contained 2,4-D, the callose grown on this tissue was devoid of measurable levels of taxanes.

CONCLUSION

Mechanical wounding with or without auxin application did not consistently induce higher taxol or baccatin III levels in *T. media* cv *Hicksii* bark, at least within three weeks. Our findings suggest that the effect of wounding on bark taxol levels depends on the phenological state or environmental conditions of the plant; furthermore, studying taxane induction in needles remains a possibility worth investigating. The results of this experiment also suggest that synthetic auxins such as 2,4-D decrease terpene concentrations.

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